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Genomic Structure and Expression of the Sea Urchin Soluble Guanylyl Cyclase β Subunit Gene

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ABSTRACT—We obtained the full-length cDNA and genomic DNA clones of *HpGCS-β_1* encoding the β subunit of soluble guanylyl cyclase (soluble GC), which is expressed in the testis and ovary of the sea urchin *Hemicentrotus pulcherrimus*. Reverse transcription-polymerase chain reaction analysis demonstrated that the *HpGCS-β_1* transcript was detected in unfertilized eggs as well as in the testis and ovary. The open reading frame predicted a protein of 604 amino acids with a putative cyclase catalytic domain conserved in membrane GCs and adenylyl cyclases. ^{105}His, ^{78}Cys, and ^{122}Cys, important amino acids for heme-binding in the β_1 subunit of soluble GCs in vertebrates, were conserved in the corresponding positions of HpGCS-β_1. The *HpGCS-β_1* gene consisted of 8 exons and had a span of 26 kbp. A comparison of genomic structure of the *HpGCS-β_1* gene with that of the soluble GC β_1 subunit gene (*OlGCS-β_1*) of the medaka fish indicated that exon 4 in the *HpGCS-β_1* gene corresponds to exons 4 to 9 in the *OlGCS-β_1* gene.
Key words: soluble guanylyl cyclase, sea urchin, genome, cDNA, *Hemicentrotus pulcherrimus*

INTRODUCTION

During animal fertilizations, an egg must produce a proper intracellular calcium signal in order to allow for development to proceed normally. Calcium release mechanisms have been extensively studied in sea urchins (Stricker, 1999). Willmott *et al*. (1996) demonstrated that nitric oxide (NO) mobilizes calcium from intracellular stores in the sea urchin egg *via* a pathway involving guanosine 3',5'-cyclic monophosphate (cGMP) and cyclic ADP-ribose (cADPR). It has been reported that NO synthase (NOS) is active in spermatozoa and eggs in the sea urchin *Strongylocentrotus purupuratus* and endogenously activated NO-associated bioactivities are necessary for successful fertilization (Kuo *et al*., 2000). Soluble guanylyl cyclase (soluble GC) is a heme-containing heterodimer composed of α and β subunits and is activated by binding of NO to a single heme associated with the β_1 subunit (Wedel *et al*., 1994). To date, many cDNA clones and several genomic DNA clones for both subunits have been obtained from various sources, *e.g.,* human (Giuili *et al*., 1992; Zabel *et al*., 1998; Behrends *et al*., 2000), bovine (Koesling *et al*., 1988, 1990), rat (Nakane *et al*., 1988, 1990; Yuen *et al*., 1990; Harteneck *et al*., 1991; Koglin *et al*., 2000), mouse (Sharina *et al*., 2000), medaka fish (Mikami *et al*., 1998, 1999), *Drosophila* (Shah *et al*., 1995), the tobacco hornworm moth *Manduca sexta* (Nighoron *et al*., 1998), nematode (Yu *et al*., 1997), and yeast (Kuo *et al*., 1998); the chemical and biological natures of these clones have been characterized. However, little is known about the soluble GC/cGMP-mediated biochemical pathway in marine invertebrates including the sea urchins. Here, we report that the sea urchin *Hemicentrotus pulcherrimus*, a very common species in Japan that is very closely related to *S. purupuratus*, possesses a soluble GC β_1 subunit of the highly conserved primary structure and the gene is expressed in the testis and ovary, as well as in unfertilized eggs. We also report that exon 4 in the β_1 subunit gene (*HpGCS-β_1*) of *H. pulcherrimus* soluble GC corresponds to exons 4 to 9 of the medaka fish β_1 subunit gene (*OlGCS-β_1*).

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MATERIALS AND METHODS

Materials

H. pulcherrimus sea urchins were collected along the coast near Noto Marine Laboratory, Kanazawa University. The gonads and gametes were obtained from mature adult animals as described previously (Xu *et al*., 2001).

Preparation of RNA and amplification of cDNA fragments by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from the *H. pulcherrimus* tissues, eggs, and embryos according to the method described by Chomczynski and Sacchi (1987). Poly(A)$^+$RNA was isolated using Oligotex-dT30-<Super> (Roche Diagnostics, Japan), according to the manufacturer's protocol.

Four degenerate oligonucleotide primers (F1, 5'-CTIMGN-CTNAARGGNCAAATGATCTAC-3'; F2, 5'-AAGACIGACAGGYTNC-TNTAYTCNGT-3'; R1, 5'-ATTICCRAANAGACARTANCGNGGCAT-3'; R2, 5'-SAGRTTSACWGTATTNCCRAANAGACA-3') were synthesized based on the amino acid sequences of 4 conserved regions (LRLKGQMIY, KTDRLLYSV, CLFGNTVNL, MPRYCLFGN) in known

soluble GCs. A set of the primers was used to amplify the cDNA fragments of *H. pulcherrimus* soluble GC from cDNA reverse-transcribed total RNA or poly(A)$^+$RNA of the *H. pulcherrimus* testis, as described previously (Seimiya *et al.*, 1997). The PCR products were purified, subcloned into the plasmid vector pBluescript II KS(–) (Stratagene, La Jolla, CA, USA), and sequenced.

(A)

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ACAACGACCGTTCTCCAACTCCGTGCATATAACACATACTCGTGTAAGCGTCACCACTCAACGAAGAGAGGTGCGCGGTCGGTTTTGCGA(  90)
AAAGTGAGATTTCATCTGTCCATTGCAACAGAACAAAAAAGAAAATTGGAATTCAGTGTTCAATTAAACTCTCGACAAAAGGGCTGCATT( 180)

                                                                                  exon1|exon2
CTGATTGTCTTCTTGGAAGTTGTGGCACCTTGAGTAGCGCGCTAAGGAAAGACAAAAAAGTCAACCAAAAATGTATGGATTCGTTAATCA( 270)

                                                                  exon2|exon3
CGCTTTGGAGCTGCTGGTTCTTCGAGAACATGGCAAAGACAAATGGGAAGAAATCAAACGCGAAGCCGCAGTGGAGATCGAAGGCAGTTT( 360)

                                                                  exon3|exon4
CTTAGTCCGAATCGTGTATGATGATGTCTTATCTTATGATCTAGTCGGAGCCGCCGTCAAAGTACTCGAAATAAGTGCCAATGATCTGTT( 450)
                                                                  tttaagatcaatgctggagacatctt( 449)
                                                                  exon3|exon4
GGAGGCATTCGGTCGGATGTTCTTTGAATTTTGCGTCGAGTCTGGCTACGACAACATCTTGAACGTCCTCGGTTCAACCACACGTCACTT( 540)
gcagatgtttgggaagatgttttttgaattttgccaggaatcaggatatgacactattttgcgtgtcttgggctccaatgttcgagaatt( 539)

CTTGCAGAACTTAGATGCTCTTCACGATCATCTTGCCTCAATCTACCCGGGAATGCGAGCACCTTCATTCAGATGCAGTACACGAGAGAG( 630)
cttgcagaatctggatgctctacacgatcacttgggtaccatttatcccggaatgagagctccctcgtttcgctgcacggatgcagagaa( 629)
  exon4|exon5
TGACGGTGCTCTTGTCCTTCACTACTACTCCGAGCGACCAGGGCTGGAGCACATCGTCATCGGGTTGGTACGCAGTGTGGCAAAGACCCT( 720)
gggaaacaatctgattcttcattactactctgagagggagggcttacaagatattgtaattggcatcattaaaactgtcgcccaacaaat( 719)

CCATGGCTCCGAAGTCCATGTCGAGATCATCAAGAATAAAGGAGAAGACTGCGATCATGTTCAGTTTGCGATCATCGAGAAG---GTGGA( 807)
ccatggaacagagatcgagatgaaggtaatccaacaaaaaagtgaagagtgcgaccacatcaagtttctgattgaagagaaggactctga( 809)
                                                  exon5|exon6
GACAGCGAAGATTGAGAAGCAGGCGCGACAGAACTTACTGGCTCTCTCCAAGGAACCCAAAATCAGTCCATCAACACTGTGCAGGATCCT( 897)
ggaggaggcgttcaatgaagaccttgatggctttgaggagaacggcactcaggagactcggatcagcccctacacgttctgcaaggcctt( 899)

GCCCTTCCACATCATGTTCAATGCTGAATTAAACGTAGAGCAAGCAGGTAACTCCATTCAAAGGATTGTACCTAACATTATCAATCCTAA( 987)
ccccttcacctcatgttcgacaaggacctcatgctcacccagtgtgggaatgccatctaccgggttctgccgcagctccagcctggcag( 989)
                                                                  exon6|exon7
CTGCAGAATGACGGACTTATTCCATATCGTGCGCCCTCACATGGAGTTCACCTTCAAGTCTATCCTCAGTCATGCCAACACCATTTACGT(1077)
ctgtatccttccatcagtgttttctttggtccggcctcatatcgacttcagttttcatggaattctttctcacataaacacggtctttgt(1079)

GCTGAAAACCAACTCTGGTGTGGTAAATCCAAACAACCCCCGTAACGGCTCC------------------ATCCCAGCACTCAAGCTCAA(1149)
tctacgcagcaaggagggcctactaaatgtggagacggtggagaatgaggatgagctaacgggggtggagatcagctgtctgagactgaa(1169)
                  exon7|exon8
AGGCCAGATGCTTCACGTACCAGAGTCCAATGTACTACTATATCTCTGTTCACCACACGTCATCAATCTTGATGAGCTGAGGCAACGTGA(1239)
agggcagatgatttacttgccagaggcagagaacattcttttttctttgctcacccagtgttatgaacttggatgatctaacaaggagggg(1279)
                                                  exon8|exon9
ACTGTACCTGAGTGATATCCCATTGCATGATGCTACGAGGGACCTGGTACTGATCTCCGAGAGGTTTGACGAGGAGTACAAACTAACCCA(1329)
gctgtacctgagtgacatcccactgcatgacgccacccgtgacctggttctgctgggtgagcagtttcgggaggagtacaagctgaccca(1369)
                                                                  exon4|exon5
GAAGCTAGAGATCCTCACAGACAAGCTACAACAGACCTACCGTGAGATCGAGAATGAAAAGAAGAAAACTGACAGGCTCCTCTATTCCAT(1419)
agagctggaaatcttgacagatcgtcttcagcacactctccgggccctggaggatgaaaaaaagaagactgatagactgc          (1449)
                                                                  exon9|exon10
CCTCCCTCCCTCCGTTGCTAACGAGTTACGTCATCATCGACCTGTTCCCGCCAAAAAGTTTGAGTGTGTGACTCTGATGTTCAGTGGGAT(1509)
CTTTGGCTTTGGAGACTTTTGTCGTCGCTACTCACACGACGCTATGAAGATCGTTAGCCTTCTTAACAGTGTTTACACCAAGTTTGACGT(1599)

                                  exon5|exon6
CCTTATGGAAAATAACCCAGACGTTTACAAGGTAGAGACAGTGGGTGATAAATACATGGCAGTAAGTGGTCTACCCGTACCATGTGCTGA(1689)

                                                                  exon6|exon7
TCATGCTAAATGTATCGCTAAGATGGCGCTTGAAATGAAGGAACTCTCTGCAGATGTCATCATGGAGGGTGACCCTATAGTGATCACTAT(1779)
TGGTGTCTACAGTGGAGAGGTGGTGACAGGAGTGGTGGGACAACGCATGCCGAGGTACTGTCTCTTTGGTAACACTGTCAACCTCACCTC(1869)

                                                  exon7|exon8
CAGGACAGAGACTACTGGTGTCACGGGCAAGATCAACATCGCCGATACTGCTTACGATTGTTTGATGGAGCCACAGAATGCAGACCCAAC(1959)
CTTCCAGTTCGATTTCCGAGGTCTAGTCAACATGAAGGGCAAGCCTAAACCCTGCCCATGCTATCTGCTCTCAAGGAAACCAGCTGAAGC(2049)
GAAACCTGAACCATAAACCTGCAGCCTAGCTCAAGTCAAGACATTAACCGCTGAATCTATCTTTCTGATCAGGGGAGACAGTGTACGGTT(2139)
TGAACTTGTCCACGAAGTACCATCAAATAAATTAAAGGGTTACACACCATTACCCTTTTAAACCTGAACCATAAATGTGCAACCTAGCTC(2229)
AAGTCAAGACATTAACCGCTGGATCTATCATTCTGATTAGAGGAGACAGTGTACGGTTTGAACTGGTCCACGAAGCACCATCAAATAAAA(2319)
TTAAAGGGTTACACTCCATTACCCTTTTAAACCTGAACCATAAATAGTCTAGCTGAAGTCAAGGCAGTAACTGCTGGATTTATCACTCTG(2409)
ATCAAGAGATACAATGTGTGGTTTGAACTGTTTCACGTAGTACCATCAACGTCAAAGGTTACACACTGTTACCCTACGGCGTTTTAACAA(2499)
AATATCCTGAAATGGTCGATATTATTGCATATGACTTTGTTAAAATTGACACGCGGTTTATATTCAAAATTAAATACTAACATGAAGCAA(2589)
ATTT                                                                                          (2593)
```

(B)

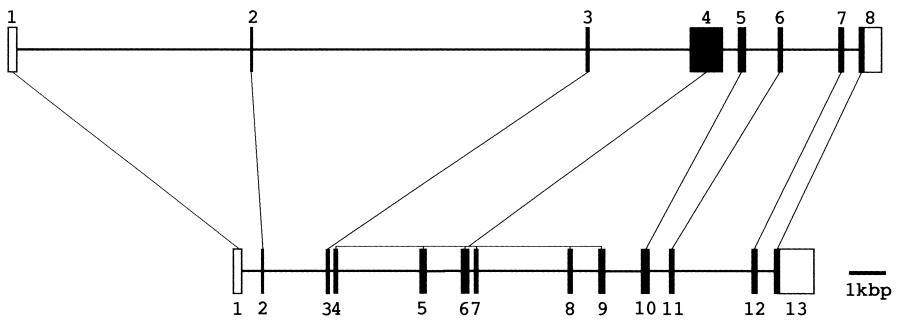


Table 1. Exon and intron sizes in the β_1 subunit genes of *H. pulcherrimus* (*HpGCS-β_1*), medaka fish (*OlGCS-β_1*), and human soluble GCs.

	Exon size (bp)			Intron size (bp)		
No.	sea urchin	medaka*	human**	sea urchin	medaka*	Human**
1	255	255	82	ca. 7000	269	643
2	72	74	74	ca. 10000	1834	15106
3	101	101	101	ca. 3000	77	2455
4	976	119	119	419	2422	12072
5	226	199	198	921	998	>3596
6	141	231	231	1643	112	1253
7	155	117	117	435	2653	919
8	667	134	134		742	3367
9		198	198		1056	2267
10		238	238		554	1044
11		141	141		2291	827
12		155	156		231	355
13		1182	127			1159
14			519			
total	2593	3144	2435	23418	13239	>45063

* Mikami *et al.*, 1998.

** from Celera Human Genome Sequence Data.

5'- and 3'-Rapid amplification of cDNA ends (5'- and 3'-RACE)

To obtain the full-length sequence of *HpGCS-β_1* cDNA, the 5'-portion of the cDNA was amplified by the 5'-RACE method (Frohman *et al.*, 1988) using the 5'-RACE System for Rapid Amplification of cDNA Ends, ver 2.0 (Life Technologies Inc.). Total RNA (1 μg) isolated from the testis was reverse-transcribed with gene-specific antisense oligonucleotide primers (GSP1-1, GSP2-1, and GSP3-1). The cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase, and amplified by PCR with the Abridged Anchor Primer (Life Technologies) and other gene-specific antisense oligonucleotide primers (GSP1-2, GSP2-2, and GSP3-2). The following PCR conditions were applied: for GSP1-2, denaturation at 95°C for 5 min followed by 35 amplification cycles (95°C for 30 sec, 66°C for 30 sec, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP2-2, denaturation at 95°C for 5 min followed by 35 amplification cycles (95°C for 30 sec, 65°C for 30 sec, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP3-2, denaturation at 95°C for 5 min followed by 35 amplification cycles (95°C for 30 sec, 67°C for 30 sec, and 72°C for 90 sec) and a final extension at 72°C for 10 min. To enrich the 5'-RACE products, one-fifteenth volume of the primary 5'-RACE products was reamplified by 25 additional cycles using the Abridged Universal Amplification Primer (AUAP; Life Technologies Inc.) and nested primers (GSP1-3, GSP2-3, and GSP3-3). Amplification was performed as follows: for GSP1-3, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 63°C for 30 sec, and 72°C for 90 sec) and a final extension at 72°C for 10 min; for GSP2-3 and GSP3-3, amplification was performed under the same conditions as used for GSP1-3. The PCR products were cloned into pBluescript II KS (+) and sequenced. The gene-specific primers used were complementary to nucleotide positions of *HpGCS-β_1* cDNA; 1649-1668 (GSP1-1), 1529-1555 (GSP1-2), 1483-1508 (GSP1-3), 1438-1459 (GSP2-1), 1354-1381 (GSP2-2), 1327-1352 (GSP2-3), 542-561 (GSP3-1), 510-538 (GSP3-2), and 480-505 (GSP3-3). The 5'-RACE products overlapped in 50–200 bp with the 5' end of the clone that had been isolated.

The 3'-portion of the cDNA was amplified by the 3'-RACE method (Frohman *et al.*, 1988) using the 3'-Full RACE Core Set (Takara Shuzo Co., Ltd., Osaka, Japan). Total RNA (1 μg) was reverse-transcribed with an Oligo dT-3'sites Adaptor Primer (Takara Shuzo). The cDNA was amplified by PCR with the 3'sites Adaptor Primer (Takara Shuzo) and another gene-specific oligonucleotide primer (GSP4-1). One-fifteenth volume of the PCR product was amplified by PCR with the 3'sites Adaptor Primer (Takara Shuzo) and another gene-specific oligonucleotide primer (GSP4-2). The following PCR conditions were used: for GSP4-1, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 61.5°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 10 min; for GSP4-2, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 61.5°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 10 min. The PCR products were cloned into pBluescript II KS (+) and sequenced. The gene-specific primers used for 3'-RACE were nucleotide positions of *HpGCS-β_1* cDNA; 1670-1691 (GSP4-1) and 1780-1801 (GSP4-2). The 3'-RACE product overlapped in 50 bp with the 3' end of the clone previously isolated.

Molecular phylogenetic analysis

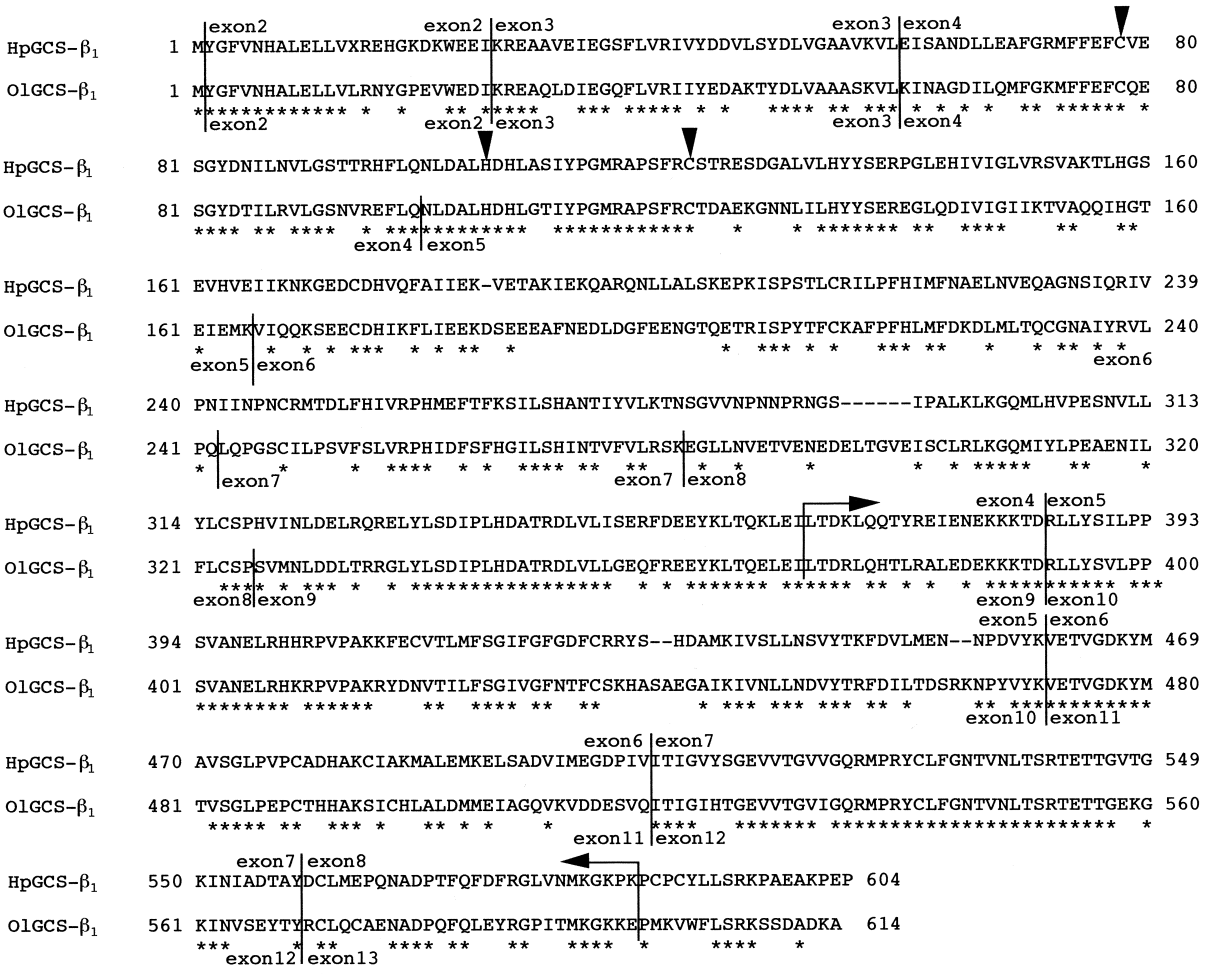
The nucleotide and deduced amino acid sequences of *HpGCS-β_1* were compared with those of known soluble GC subunits using the Clustal W program (Thompson *et al.*, 1994) and the sequence editor SeqPub (Gilbert, Indiana University, Indianapolis, USA). The unrooted phylogenetic tree was constructed using the aligned amino acid sequences of the catalytic domain of various soluble GCs by the neighbor-joining algorithms in the PROTRAS program of PHYLIP (version 3.572) (Felsenstein, 1989) and the Clustal W program (Saitou and Nei, 1987).

Isolation and sequencing of genomic DNA encoding HpGCS-β_1

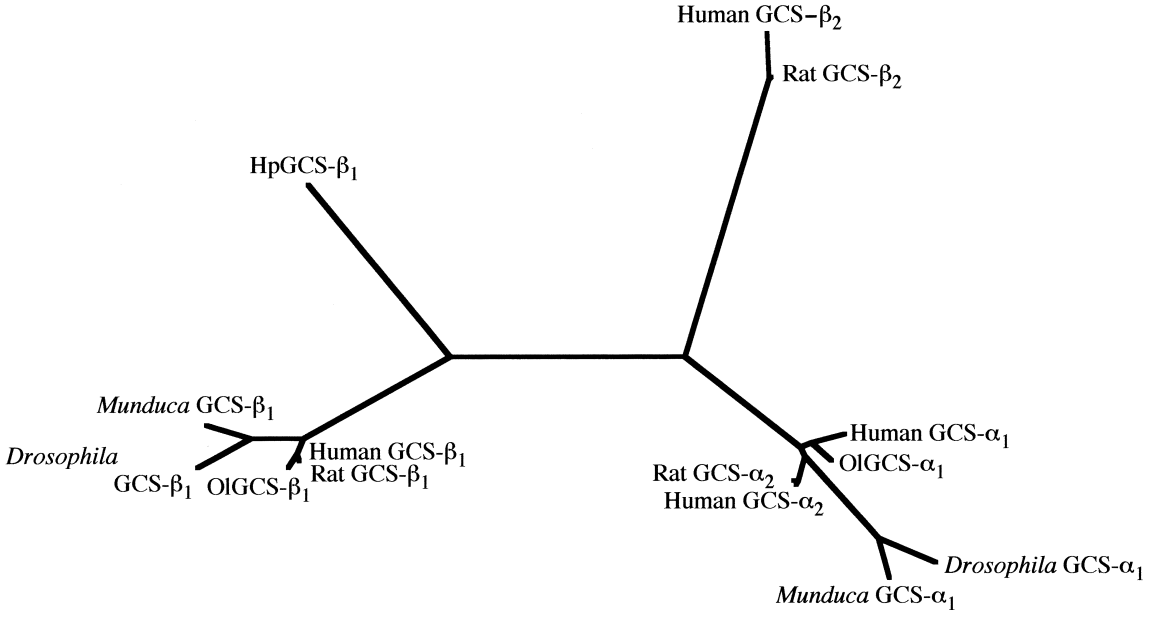
Genomic DNA was isolated from the *H. pulcherrimus* testis by the procedure described previously (Mikami *et al.*, 1998). In order to obtain a genomic DNA fragment containing an intron and a portion of

Fig. 1. The nucleotide sequence of *HpGCS-β_1* cDNA (A) and the schematic exon/intron organization of the *HpGCS-β_1* gene (B). (A) The nucleotide sequence is indicated by a single-letter code (capital letters for *HpGCS-β_1* cDNA, small letters for *OlGCS-β_1* cDNA). Gaps in the sequences are indicated by a dash (–). The location of introns is indicated by vertical lines with exon numbers. The numbers on the right side indicate the nucleotide sequence numbers. Nucleotides with open boxes denote a termination or initiation codon. (B) Exons for coding regions are indicated by closed boxes with numbers; horizontal lines denote introns. Open boxes indicate exons for an UTR. The upper panel represents the *HpGCS-β_1* gene with 8 exons and the lower panel represents the *OlGCS-β_1* gene with 13 exons. Exon(s) in the *HpGCS-β_1* gene corresponding to exon(s) in the *OlGCS-β_1* gene are joined by lines.

(A)


exon2 exon2 exon3 exon3 exon4
HpGCS-β1 1 MYGFVNHALELLVXREHGKDKWEEIKREAAVEIEGSFLVRIVYDDVLSYDLVGAAVKVLEISANDLLEAFGRMFFEFCVE 80
OlGCS-β1 1 MYGFVNHALELLVLRNYGPEVWEDIKREAQLDIEGQFLVRIIYEDAKTYDLVAAASKVLKINAGDILQMFGKMFFEFCQE 80
exon2 exon2 exon3 exon3 exon4
HpGCS-β1 81 SGYDNILNVLGSTTRHFLQNLDALHDHLASIYPGMRAPSFRCSTRESDGALVLHYYSERPGLEHIVIGLVRSVAKTLHGS 160
OlGCS-β1 81 SGYDTILRVLGSNVREFLQNLDALHDHLGTIYPGMRAPSFRCTDAEKGNNLILHYYSEREGLQDIVIGIIKTVAQQIHGT 160
exon4 exon5
HpGCS-β1 161 EVHVEIIKNKGEDCDHVQFAIIEK-VETAKIEKQARQNLLALSKEPKISPSTLCRILPFHIMFNAELNVEQAGNSIQRIV 239
OlGCS-β1 161 EIEMKVIQQKSEECDHIKFLIEEKDSEEEAFNEDLDGFEENGTQETRISPYTFCKAFPFHLMFDKDLMLTQCGNAIYRVL 240
exon5 exon6 exon6
HpGCS-β1 240 PNIINPNCRMTDLFHIVRPHMEFTFKSILSHANTIYVLKTNSGVVNPNNPRNGS------IPALKLKGQMLHVPESNVLL 313
OlGCS-β1 241 PQLQPGSCILPSVFSLVRPHIDFSFHGILSHINTVFVLRSKEGLLNVETVENEDELTGVEISCLRLKGQMIYLPEAENIL 320
exon7 exon7 exon8
exon4 exon5
HpGCS-β1 314 YLCSPHVINLDELRQRELYLSDIPLHDATRDLVLISERFDEEYKLTQKLEILTDKLQQTYREIENEKKKTDRLLYSILPP 393
OlGCS-β1 321 FLCSPSVMNLDDLTRRGLYLSDIPLHDATRDLVLLGEQFREEYKLTQELEILTDRLQHTLRALEDEKKKTDRLLYSVLPP 400
exon8 exon9 exon9 exon10
exon5 exon6
HpGCS-β1 394 SVANELRHHRPVPAKKFECVTLMFSGIFGFGDFCRRYS--HDAMKIVSLLNSVYTKFDVLMEN--NPDVYKVETVGDKYM 469
OlGCS-β1 401 SVANELRHKRPVPAKRYDNVTILFSGIVGFNTFCSKHASAEGAIKIVNLLNDVYTRFDILTDSRKNPYVYKVETVGDKYM 480
exon10 exon11
exon6 exon7
HpGCS-β1 470 AVSGLPVPCADHAKCIAKMALEMKELSADVIMEGDPIVITIGVYSGEVVTGVVGQRMPRYCLFGNTVNLTSRTETTGVTG 549
OlGCS-β1 481 TVSGLPEPCTHHAKSICHLALDMMEIAGQVKVDDESVQITIGIHTGEVVTGVIGQRMPRYCLFGNTVNLTSRTETTGEKG 560
exon11 exon12
exon7 exon8
HpGCS-β1 550 KINIADTAYDCLMEPQNADPTFQFDFRGLVNMKGKPKPCPCYLLSRKPAEAKPEP 604
OlGCS-β1 561 KINVSEYTYRCLQCAENADPQFQLEYRGPITMKGKKEPMKVWFLSRKSSDADKA 614
exon12 exon13

(B)


Human GCS-β2
Rat GCS-β2
HpGCS-β1
Munduca GCS-β1
Drosophila GCS-β1
Human GCS-β1
Rat GCS-β1
OlGCS-β1
Human GCS-α1
OlGCS-α1
Rat GCS-α2
Human GCS-α2
Drosophila GCS-α1
Munduca GCS-α1

exons, we performed PCR using *H. pulcherrimus* testis genomic DNA (10 μg) as a template and various gene-specific primers (EX1, EX2-1, EX2-2, EX3-1, EX3-2, EX4-1, EX4-2, EX5, EX6, EX7, EX8, GSP3-1, GSP4-1, and GSP-4-2). These primers were synthesized based on the nucleotide sequence of *HpGCS-β_1* cDNA. The following PCR conditions were applied: for EX1 and EX2-2, denaturation at 95°C for 5 min followed by 30 amplification cycles (98°C for 20 sec and 61.5°C for 5 min) and a final extension at 72°C for 10 min; for EX2-1 and EX3-2, denaturation at 95°C for 5 min followed by 30 amplification cycles (98°C for 20 sec and 61.5°C for 5 min) and a final extension at 72°C for 10 min; for EX3-1 and EX3-2, denaturation at 95°C for 5 min followed by 30 amplification cycles (98°C for 20 sec and 64°C for 8 min) and a final extension at 72°C for 10 min; for EX4-1 and GSP3-1, and EX5 and EX6, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 63°C for 30 sec, and 72°C for 2 min) and a final extension at 72°C for 10 min; for GSP4-1 and EX7, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 30 sec, 61.5°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 10 min; for GSP4-2 and EX8, denaturation at 95°C for 5 min followed by 30 amplification cycles (95°C for 5 min, 62°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 10 min. The PCR products were subjected to sequencing directly or after subcloning into pBluescript II KS(+). The gene-specific primers used were complementary to the nucleotide positions of *HpGCS-β_1* cDNA; 37-60 (EX1), 257-285 (EX2-1), 294-320 (EX2-2), 338-361 (EX3-1), 397-420 (EX3-2), 889-911 (EX4-1), 935-961 (EX4-2), 1440-1466 (EX5), 1728-1754 (EX6), 1882-1907 (EX7), and 2465-2493 (EX8).

Other methods

The nucleotide sequence was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) with an Applied Biosystem 377 sequencer or a 3100 Genetic Analyzer. Data was analyzed with DNASIS software (Hitachi Software Engineering Co., Yokohama, Japan) and GENETYX-MAX/version 7.2.0. (Software Development, Tokyo, Japan).

RESULTS AND DISCUSSION

As shown in Fig. 1 and Table 1, the *HpGCS-β_1* cDNA consists of a 250-bp 5' untranslated region (UTR), a 2012-bp open reading frame (ORF), and a 531-bp 3'-UTR. The *HpGCS-β_1* gene spans over 26 kbp and consists of 8 exons. The GT-AG rule was conserved for all splice sites. The sizes of all introns in the *HpGCS-β_1* gene are larger than those in the medaka fish soluble GC β_1 subunit gene *OlGCS-β_1* and some are larger than those in the human soluble GC β_1 subunit gene. Exons 1 to 3 in the *HpGCS-β_1* gene are the same size as the corresponding exon in the *OlGCS-β_1* gene. However, exon 4 in the *HpGCS-β_1* gene is much larger than that of the *OlGCS-β_1* gene and the human soluble GC β_1 subunit gene and corresponds to the nucleotides together with exons 4 to 9 in the *OlGCS-β_1* gene, suggesting that intron insertion occurred during gene evolution from invertebrates to vertebrates. Although there is little difference according to size, exons 5 to 8 of the *HpGCS-β_1* gene seem to correspond to exons 10 to 13 in the *OlGCS-β_1* gene, respectively.

The ORF of *HpGCS-β_1* cDNA encodes 604 amino acids (Fig. 2). Comparison of the deduced amino acid sequence of *HpGCS-β_1* with that of each subunit of known soluble GCs indicated that the entire amino acid sequence of HpGCS-β_1 was 57.1, 57.9, and 58.8% identical to that of human, medaka fish, and *Manduca sexta* GCS-β_1, respectively. Considering that the entire amino acid sequence of the soluble GC β_1 subunit is highly conserved among vertebrates (*i.e.,* the identity throughout the entire protein between humans and rats is 98.5%, and that between humans and the medaka fish is 89.8%). The identity in the amino acid sequence between the sea urchin and other animals was rather low. However, the amino acid sequence identity in the catalytic domain of the α_1, α_2, β_1, or β_2 subunit between sea urchin and other animals was about 46, 45, 63, and 42%, respectively. Furthermore, phylogenetic analysis demonstrated that HpGCS-β_1 phylogenetically separates from the α_1, α_2, or β_2 subunit cluster and was closer to the β_1 subunit cluster of vertebrate soluble GC than to soluble GC of the insects, *Drosophila* and *Manduca* (Fig. 2B). In the b_1 subunit of vertebrate soluble GC, ^{105}His, ^{78}Cys, and ^{122}Cys have been demonstrated to be important amino acids for heme-binding (Stone and Marletta, 1994; Wedel *et al.*, 1994; Foerster *et al.*, 1996). These amino acid residues are conserved in the corresponding positions of HpGCS-β_1, respectively (Fig. 2A). All of these studies suggest that HpGCS-β_1 is a heme-containing *H. pulcherrimus* soluble GC β_1 subunit and can be activated by NO after association with its proper counterpart α_1 subunit. In this regard, it should be mentioned that sea urchin gametes contain a NO synthase and NO; sea urchin gamete NO-related bioactivity appears to be critical for egg activation, yet its mechanism remains unknown (Kuo *et al.*, 2000).

Our results demonstrated that the *HpGCS-β_1* gene was expressed in the testis and ovary of *H. pulcherrimus* and its transcript was also detected in the unfertilized egg as well as in the embryo (Fig. 3). This finding is consistent with our previous results demonstrating 1) that the gene transcripts of both subunits of medaka fish soluble GC were present in unfertilized eggs, and 2) that these transcripts were temporarily reduced immediately after fertilization and then subsequently increased to previous levels (Mikami *et al.*, 1998). However, at present the point at which the *HpGCS-β_1* gene is transcribed during oogenesis remains unknown. It also remains unclear how the transcript is stored in the unfertilized egg and whether active soluble GC is present in the unfertilized egg. We believe that the pathway involving cGMP and cADPR in calcium

Fig. 2. Alignment of the amino acid sequences of HpGCS-β_1 and OlGCS-β_1 (A) and molecular phylogenetic analysis using amino acid sequences of the cyclase catalytic domain of known soluble GC β_1 subunits (B). (A) The deduced amino acid sequence of *HpGCS-β_1* was compared with that of *OlGCS-β_1*. The amino acid sequence between the arrows denotes the cyclase catalytic domain. Identical amino acids between HpGCS-β_1 and OlGCS-β_1 are indicated with asterisks (*) below the residues. Gaps in the sequences are indicated by a dash (–). The conserved histidine and cysteine residues necessary for heme-binding are indicated by arrowheads. (B) Aligned amino acid sequences of the catalytic domain of soluble GC α and β subunits of human (Zabel *et al.*, 1998; Behrends *et al.*, 2000), rat (Nakane *et al.*, 1988, 1990; Yuen *et al.*, 1990; Harteneck *et al.*, 1991; Koglin *et al.*, 2000), medaka fish (Mikami *et al.*, 1998), *Drosophila melanogaster* (Shah *et al.*, 1995), and *Munduca sexta* (Nighoron *et al.*, 1998) were subjected to phylogenetic analysis. Branch lengths were proportional to evolutionary distances.

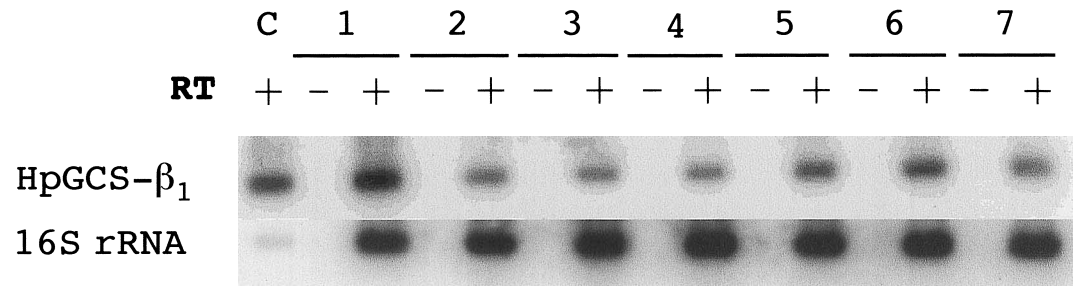


Fig. 3. RT-PCR analysis of *HpGCS-β_1* transcripts in the *H. pulcherrimus* adult tissues, eggs, and embryos. The *HpGCS-β_1* transcript was detected by RT-PCR in the testis (1), ovary (2), unfertilized eggs (3), embryos directly after fertilization (4), 2-cell stage embryos (5), 4-cell stage embryos (6), and 8-cell stage embryos (7). The 16S ribosomal RNA was amplified as an internal control. RT + and RT – represent amplification with and without reverse transcriptase, respectively.

release upon fertilization might play an important role in this process (Willmott *et al.*, 1996). However, several conflicting papers have been published on the role of a cGMP-signaling pathway in animal fertilization (Ciapa and Epel, 1996; Lee *et al.*, 1996). The present study of NO-activatable soluble GC in sea urchins is important for understanding the mechanisms of action during the early phase of animal fertilization. Further study on *H. pulcherrimus* soluble GC is currently being conducted in our laboratory.

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